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PARTIAL CHARACTERIZATION OF L- -GLYCEROPHOSPHATE DEHYDROGENASE --ETC(U)
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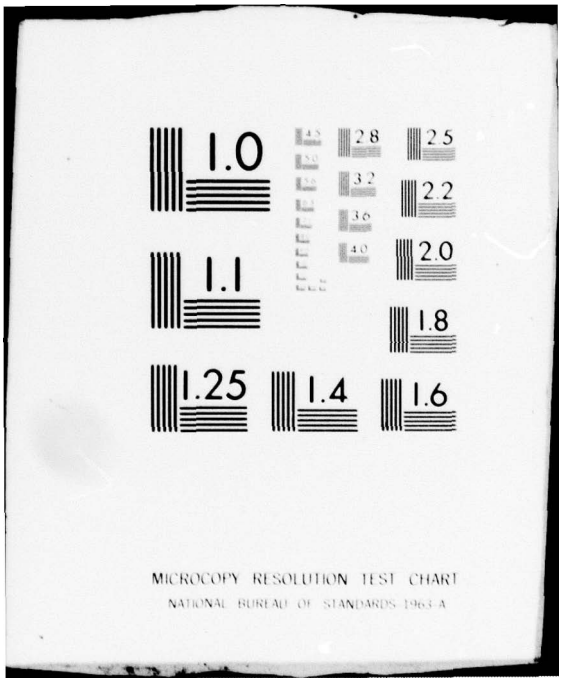
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(6) Partial Characterization of L-α-glycerophosphate Dehydrogenase from Monomorphic Trypanosoma brucei*

(9) Master's thesis,

(10) Jane H. Frey

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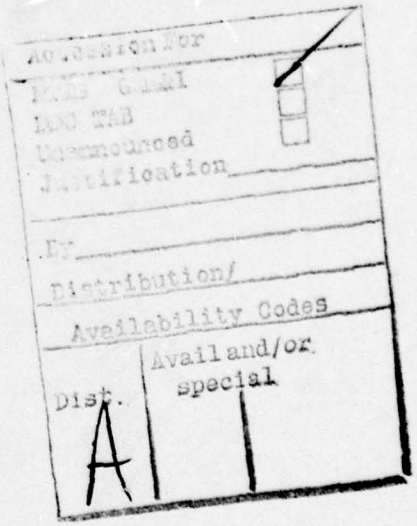

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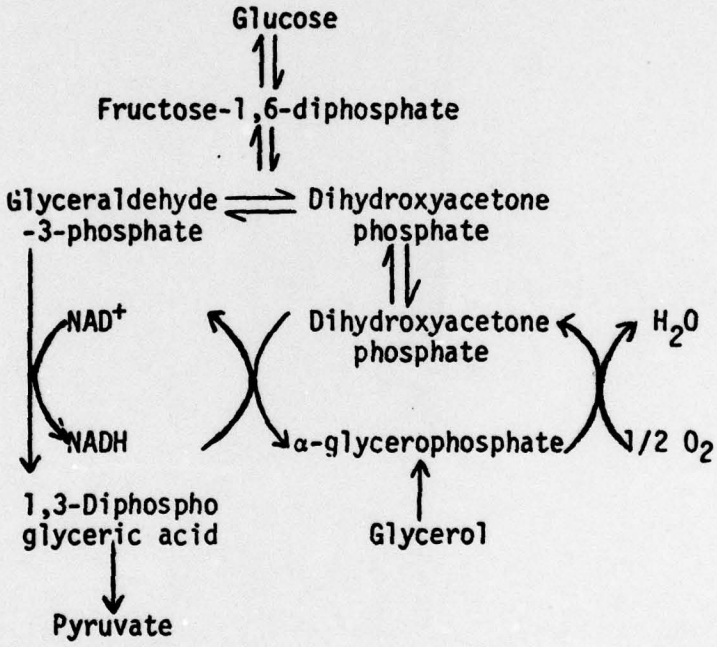
INTRODUCTION

An aspect of the host-parasite relationship of importance in the chemotherapy of trypanosomiasis concerns the biochemical differences which exist between the pathogenic bloodstream forms of trypanosomes and the tissues of their vertebrate hosts. Two important differences are that the bloodstream forms of many trypanosomes do not contain detectable cytochrome pigments nor an active mitochondrial oxidative phosphorylation (O.P.) system. Despite the lack of an oxidative-phosphorylating system, trypanosomes of the evansi-brucei subgroups are characterized by an extremely rapid but incomplete oxidation of glucose (Von Brand, 1951). This rate can be compared with the markedly lower rates for many mammalian tumor cells (Arsenberg, 1961) and the appreciably lower rates of most normal mammalian tissues. Trypanosomes consume one mole of oxygen per mole of glucose utilized. The major end products of trypanosome metabolism appear to be pyruvate together with a trace of glycerol. Utilization of ^{14}C labeled glucose by Trypanosoma rhodesiense revealed that pyruvate was formed by the glycolytic pathway and that neither the Tricarboxylic acid (TCA) cycle nor the pentose phosphate shunt were of metabolic significance in trypanosomes (Grant & Fulton, 1957). The formation of pyruvate rather than lactate and the absence of an O.P. system for the direct oxidation of reduced nicotinic adenine dinucleotide (NADH) poses a problem as to the mechanism of reoxidation of NADH formed in the glyceraldehyde-3-phosphate dehydrogenase reaction of glycolysis. Oxidation of NADH, as detected by formazan deposits, was observed in extra-mitochondrial bodies throughout the cytoplasm of the bloodstream form (Ryley, 1964). This

cytochemical evidence suggested that the oxidation of NADH might be mediated in an indirect manner by a coupled enzyme system, namely: L-α-glycerphosphate dehydrogenase and L-α-glycerophosphate oxidase. L-α-glycerophosphate dehydrogenase is an NAD-linked enzyme catalyzing the interconversion of L-α-glycerophosphate oxidase transfers hydrogen directly from L-α-glycerophosphate to atmospheric oxygen yielding dihydroxyacetone phosphate as seen in the following metabolic scheme:



Since this cycle does not appear to be associated with the oxidative phosphorylation of adenosine diphosphate (ADP), the net energy gain for this oxidative metabolism of glucose to pyruvate is two moles of adenosine triphosphate (ATP) formed per mole of glucose utilized. However, in mammalian systems approximately 15 times this amount of ATP is formed through the use of a terminal respiratory system. It is in the reliance upon the metabolic scheme noted above that a significant difference is to be found between the trypanosome parasites and their mammalian hosts. This thesis reports an

attempt to purify and characterize L-α-glycerophosphate dehydrogenase from a laboratory strain of Trypanosoma brucei.

L-α-glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8.) was first detected in rat muscle by Von Euler & Gunther in 1936, and isolated and crystallized from rabbit muscle by Baranowski (1949). The role of this enzyme in cell metabolism remained largely unexplained until high enzyme activities were found in the flight muscles of insects and in mammalian muscles (Marquardt & Brosemer, 1966). Thereafter, a key role for L-α-glycerophosphate dehydrogenase in respiration was postulated. Flight muscle mitochondria of Locusta migratoria show high respiratory rates with L-α-glycerophosphate. A system is present for the transfer of hydrogen from extra-mitochondrial NADH to the respiratory chain in Locust flight muscle mitochondria consists of L-α-glycerophosphate dehydrogenase in the cytosol and L-α-glycerophosphate dehydrogenase in the cytosol and L-α-glycerophosphate oxidase in the mitochondrion. Another system in which L-α-glycerophosphate dehydrogenase participates is the α-glycerophosphate-pyruvate dismutation reaction characterized in muscle homogenates, a pathway present in insect muscles apparently devoid of lactate dehydrogenase. Under conditions of inadequate oxygen supply a partial consumption of NADH with formation of α-glycerophosphate may prevent lowering of the redox potential of extra-mitochondrial NAD-linked systems.

The purification and crystallization of L-α-glycerophosphate dehydrogenase from bee thoracic muscles was demonstrated to be fairly easy to accomplish in contrast to isolation procedures reported for other enzymes. The isolation scheme consists of a series of ammonium sulfate fractionations. Studies on the L-α-glycerophosphate dehydro-

genase from Trypanosoma brucei attempt to correlate the properties of this enzyme with metabolically similar enzymes reported for other organisms. According to a variety of morphological and biochemical criteria, parasitologists agree that T. gambiense and T. rhodesiense (species pathogenic to man) are derived from Trypanosoma brucei. The findings noted here are compared with recent investigations on glycolytic enzymes reported by Seed & Risby (1970).

Since the other member of the coupled reaction pair, L-α-glycerophosphate oxidase, has been studied polarographically in members of the T. brucei subgroup (Grant & Sargent, 1957, 1960), it was thought that some further understanding of this cycle might be achieved by investigating the properties of L-α-glycerophosphate dehydrogenase in these organisms. One difficulty in these studies is that the monomorphic bloodstream forms of T. brucei cannot be grown nor maintained in the laboratory in culture. Rather, these organisms are grown and maintained by syringe passage, principally in laboratory rodents. By contrast, polymorphic bloodstream forms of T. gambiense and T. equiperdum will grow in culture, though only as the crithidial forms. These are morphologically distinct forms which are not transmissible by the tse-tse fly nor infections to it. Another objective of these studies was to determine whether a further understanding of the metabolism of glucose in the system employed would provide some leads to the development of a culture medium to support the growth of these organisms in vitro. Also, information on the chemical composition and enzyme function(s) of trypanosomes could contribute to our understanding of possible receptor(s) for chemotherapeutic agents. However, it is to be noted that selective inhibition of this metabolic cycle in

member organisms of this subgroup could be of limited value, since Trypanosoma rhodesiense has been shown to survive under anaerobic conditions for at least a few hours-(Grant & Fulton, 1957); the reported survival under anaerobic conditions implies that these organisms may be able to regenerate NAD for NADH by other means.

MATERIALS AND METHODS

Isolation of trypanosomes

Infections with trypanosomes were maintained by syringe passage of the organisms into laboratory mice every 48 hours. Male rats, age 6-8 wks. (National Laboratory NLR strain) of approximately 175-200 g. were injected with 2 X 10^6 trypanosomes (EATRO 691A) in a volume of 0.2 ml. of blood and diluent.

At 72 hours post infection the rats showed a parasitemia level of approximately 75 125 X 10^7 trypanosomes per ml. of blood as determined by hemocytometry. At this time the rats were bled by cardiac puncture or blood was released into the pericardial cavity using Heparin (100 units) as anticoagulant. Blood released into the pericardial cavity was collected by addition of buffer (0.041 M Tris, 0.011 M Glucose, 0.0107 M EDTA, 0.07 M NaCl, 0.0027 M KCl pH 7.5). The blood-buffer mixture was centifuged at 650 X g for 15 minutes (at 4°C) in a Sorvall RC2-B centrifuge (equipped with a swinging bucket rotor, HB-4). The centrifuged rat blood separates into three distinct layers as seen in Figure 1. The lower packed layer contains erythrocytes on top of which is the buffy coat containing trypanosomes and some white blood cells and platelets; the upper layer is serum. The upper serum layer is carefully removed by pipette and a small quantity of pH 7.5 buffer is added so as not to disrupt the trypanosome layer. The trypanosomes were suspended in the buffer by gently swirling a closed hooked tip pasteur pipette near the top of the trypanosome containing a layer, but without any mixing of the red cell material with the trypanosome layer. The trypanosomes are removed, washed twice in Tris-glucose

buffer by pelleting in a centrifuge, and resuspended after the second wash in fresh buffer. The trypanosomes are separated from other contaminated cell elements by passage through a DEAE-Cellulose column (capacity 0.85 meg./gm) eluted with Tris-glucose buffer (Lanham, 1968). Trypanosomes are collected in the eluate whereas the contaminants adhere to the gel. The purified trypanosomes thus obtained are tightly pelleted by centrifugation at 3300 x g (4,500 rpm.) for 15 mins. The supernatant is then removed and the trypanosomes are resuspended in .1 M Tris, .1 M histidine, 10 mM EDTA buffer, pH 7.0. The trypanosomes are washed 2 times in this buffer and homogenized in a Sorvall Omnimix (micro-attachment) at 7,500 rpm. for 1 min. using 200 μ glass beads in a 20% w/v solution (i.e. 1 part beads plus 5 parts liquid ≠ 20% w/v solution). Cell breakage was routinely checked by microscopy. All steps in the isolation procedure were carried out at 0-4°C.

Assays

l-α-glycerophosphate dehydrogenase (E.C. 1.1.1.8.) was assayed according to the method of Marquardt and Brosemer (1966). The spectrophotometer cuvettes contained in a volume of 1 ml: 50 mM histidine, 50 mM Tris, 5 mM EDTA buffer pH 6.6, .18 mM NADH, .5 mM DHAP (substrate) and sample. It is to be noted that the enzyme cannot be assayed at saturating levels of dihydroxyacetone phosphate (1 mM) due to substrate inhibition. Disappearance of NADH absorbance at 340 nm was recorded at 29°C. The order of addition components to the cuvette was: (i) 0.9 ml TEH (Tris-EDTA-Histidine) buffer, (ii) 25 μl NADH (absorbance checked after this addition), (iii) 50 μl of preparation to be assayed for enzyme (check for endogenous reaction) and finally

(iv) 25 µl substrate to start the reaction.

In the reverse reaction 25 µl of NAD was used and the substrate added was L-α-glycerophosphate. All enzyme assays were performed at a minimum of two enzyme concentrations i.e., the rate of the reactions were directly proportional to the concentration of enzyme used. Each experiment was performed in duplicate. A unit of enzyme activity is defined as the amount of enzyme catalyzing the disappearance of one micromole (µM) of substrate per minute per sample volume (25 µL or 50 µL).

All enzyme assays except the drug assays were performed in a Gilford model 240 Spectrophotometer at 29°C. Drug assays were done on a Cary model 15 recording spectrophotometer. Protein was determined by the method of Lowry et. al. (1951) with bovine serum albumin as the standard.

Ammonium Sulfate Fractionation

Trypanosome homogenates were prepared as described above. A supernatant fraction obtained by centrifugation of the homogenate at 9,500 x g for 15 mins. was subjected to four sequential ammonium sulfate fractionations (25%, 50%, 75%, and 100%) by addition of solid ammonium sulfate over a three hour period for each fraction as calculated from the following equation.

$$X = \frac{53.3\ (S_2 - S_1)}{1 - 0.3\ S_2}$$

x = grams of solid $(NH_4)_2\ SO_4$/100 ml. solution
S_1 = initial saturation
S_2 = final saturation
(see Kunitz, 1952)

The fractionation was carried out in a crushed ice bath and the ammonium sulfate was added with continuous stirring. When each fractional level was reached, the solution was incubated without

stirring for 1 hour to allow maximal precipitation of proteins. The solution was centrifuged for 30 mins. at 16,000 x g to remove the precipitates. The precipitates were resuspended in a minimum volume and assayed for enzyme activity using the standard procedure.

Enzyme Localization

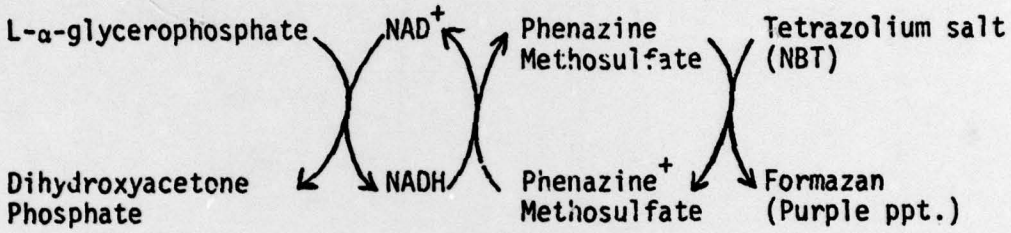
Initially, homogenates were subjected to differential centrifugation at 9500 and at 32,000 x g and the supernatants were assayed for activity. In an alternate procedure, homogenates were centrifuged at 100 x g, 4000 x g, 7800 x g, 10,000 x g and 15,000 x g. The 9500 x g pellet was treated with Triton X-100 (10 l of a 5% solution/ ml of pellet) for 5 minutes.

Electrophoresis

The technique employed was essentially that described by the Cancalco Corp. but modified to include the use of 14 X 9 cm. buffer trays, and 7 mm X 12.5 cm. tubing. The buffer used in the electrophoresis of the enzyme preparation(s) consisted of .025 M Tris and 10% glycerin (pH 8.3). Gel solutions were either 10 or 12.5% acrylamide. Electrophoresis was carried out in the cold (4°C) at 135.0 volts for 18 hours at an amperage of 2-3 milliamps per gel tube. Sample application was made by layering the protein over a stacking gel consisting of 3.5% acrylamide plus .0625% bis-acrylamide. All gels were run in duplicate.

After electrophoresis the gels were incubated in 1% Naphthol blue-black stain in 7% acetic acid for one hour, then destained under continuous stirring in 7% acetic acid for 48 hours at room temperature. The gels wre scanned at 280 nm in a Gilford Linear Transport carrier (Model 2410).

Each identically prepared gel was incubated for 1 hour in Nitroblue Tetrazolium (NBT) stain (50 mg. NAD, 5 mg. NBT, 2.5 mg. Phenazine methosulfate, 0.1 M α-glycerophosphate to a volume of 100 ml.) in the dark. The incubation of these gels was similar to the NBT staining procedure except that the reaction was carried out in dark, tightly sealed, test tubes. The mechanism of the NBT staining reaction is as follows:



At the conclusion of the one hour incubation the gel was scanned spectrophotometrically. Evidence of a non-specific dehydrogenase reaction with the Nitroblue Tetrazolium stain was determined by employing the same staining reaction but without inclusion of the specific substrate, (α-glycerophosphate). Under these conditions no staining reaction was observed.

Isoelectric focusing

The isoelectric focusing technique used was essentially that of Eder (1972). The focusing was carried out in a disc-gel electrophoresis apparatus. The gel mixture contained the following components: 3% ampholyte range pH 3-10 or range pH 5-8, 4.6% acrylamide w/v, 0.024% w/v TEMED and 0.6% ammonium persulfate. 0.05 M sulfuric acid was used as the anode solution and 0.3 M sodium hydroxide as the cathode solution. An ampholyte layer of 4% carrier ampholyte and 5% sucrose was placed on top of the gel and electro-focusing was carried out in the cold (4°C). A current of 2 milliamperes per tube, 135 v

was passed for 30 minutes to remove polymerization catalysts. Thereafter, a sample solution of 6% ampholyte, 4% sucrose and sample (protein between 125-175 µg) in a volume of 25 µl was layered on top of the gels but under the ampholyte layer with a microliter pipette. The voltage and current were restored and the gels were electrophoresed for 18 hours during which time the amperage dropped to 0.2 milliamperes per tube. At the end of 18 hours one half of the gels were stained in Nitroblue Tetrazolium stain. The duplicate gels were measured and cut into 4 mm segments by use of a device with parallel mounted razor blades. The ampholytes in the gel segments was eluted into 1 ml. of water for one hour. The pH values on the eluted extracts were recorded on a Radiometer BmS_2mk_2 Acid-Base Analyzer. From these data, a pH gradient was constructed. After the pH values were obtained from the extracts, three mls. of Nitroblue Tetrazolium stain were added to each test tube containing a gel slice and incubated in the dark overnight. The isoelectric point was calculated from both the whole gel and the stained segments and identical results were obtained with each duplicate sample.

Assays to determine the pH curve of enzyme activity utilized the standard assay procedure except that the pH of the TEH buffer was altered with either hydrochloric acid or sodium hydroxide and the enzyme was allowed to incubate in the buffer for five minutes before the reaction was started.

Assays to determine the temperature curve of enzyme activity were standard, except that the samples were preincubated for five minutes at the appropriate temperatures and then assayed.

Determination of the Michaelis-Menten constant (Km) utilized the

same assay procedure noted above except that the amount of substrate (dihydroxyacetone phosphate) was varied.

To determine the inhibitory effect of sulfhydryl reagents on enzyme activity, the reagent, NADH, and sample were incubated for 10 mins. before the reaction was started.

Reagents

Trishydroxymethylamino methane (Tris), Dihydroxyacetone phosphate (Dimethylketal Di-monocyclohexylamine salt), N-Ethylmaleimide (NEM), Iodoacetic acid (IAA), p-Hydroxymercuribenzoate (PHMB), reduced Gluthathione (GSH), DEAE-Cellulose, Nitroblue Tetrazolium, Phenazine Methosulfate, β-Diphosphopyridine Nucleotide (NAD, NADH), and α-glycerophosphate were obtained from Sigma Chemical Co. Ammonium persulfate Acrylamide, N,N'-Methylene Bisacrylamide, N,N,N',N'-Tetramethylethylenediamine (TEMED), and riboflavin were obtained from Eastman Kodak. Ammonium sulfate was obtained from Baker. Heparin was purchased from Scientific Products Co. and Ampholine pH 3-10, pH 5-8 from LKB, Sweden. All chemicals were of reagent grade.

RESULTS

Differential centrifugation of cell-free homogenates at different gravitional forces did not permit localization of enzyme activity in a discrete fraction (see Table 1). Cell-free preparations of Trypanosoma brucei exhibit L-α-glycerophosphate dehydrogenase activity in both the particulate and soluble fractions. The particle bound enzyme activity of the low speed, 9500 x g sediment material was partially released from the sediment upon treatment with Triton X-100 for 5 mins. The soluble fractions (9500 x g and 32,000 x g) exhibited specific activities similar to that obtained for the particlulate fractions at 9500 x g.

It remains uncertain as to whether the presence of enzyme in both the soluble and particulate fractions reflects enzyme compartmentation or is a manifestation of the isolation method. Since the release of L-α-glycerophosphate dehydrogenase from the particulate fraction(s) occurs upon vigorous homogenization, the enzyme may be naturally present in the cell in both a soluble and particulate state. It was seen that the application of various amounts of homogenization to isolated organisms at different stages of parasitemia produced different particle/soluble enzyme activity ratios. Likewise, the marked release of enzyme activity into a soluble-fraction upon treatment with a non-ionic detergent (Triton X-100) suggests that two distinct enzyme activities are differently localized in situ, one that is particle bound (but releaseable by various solubilization methods) and a second that represents the soluble enzyme (within the cytosol).

Partial purification of the enzyme was achieved by the use of

ammonium sulfate precipitation. Supernatant fractions were obtained by differential centrifugation of the cell homogenate at 9500 x g (see Table 2). Supernatant fractions were subjected to four ammonium sulfate fractionations. Each sequential fraction was assayed for specific activity and contains approximately 35% of the enzyme from the homogenate or 66% from the 9500 x g supernate. The enzyme specific activity in the 50% ammonium sulfate fraction represents a 15.9 fold purification compared to the initial homogenate, and an 11.8 fold purification over the supernatant fraction. The several fold purification of L-α-glycerophosphate dehydrogenase by ammonium sulfate was also obtained for bee L-α-glycerophosphate dehydrogenase where crystallization of the enzyme occurred merely by the use of an ammonium sulfate precipitation procedure (Marquardt and Brosemer, 1960).

Enzyme purity of the 50% ammonium sulfate fraction was determined by polyacrylamide disc gel electrophoresis. Three closely associated bands are seen on the 10% and 12.5% gels. The 12.5% gels offer the best resolution. Spectrophotometric scans and gel photographs are seen in Figures 2 and 3. Correspondence between protein band(s) and enzyme band(s) localization was achieved by the use of two stains, an enzyme specific stain (Nitroblue Tetrazolium) as well as a general protein stain (Napthol blue black). The polyacrylamide gels reveal that the 50% ammonium sulfate 'cut' contains purified enzyme plus some contaminating proteins. A faint staining region of contaminating proteins in the Napthol blue black gels is seen to the right of the enzyme band(s). This is significant in that hexokinase, aldolase and acid phosphatase from T. rhodesiense are known to precipitate within a range of 45-75% ammonium sulfate saturation (Seed and Risby, 1969).

It is possible that the contaminating proteins may be other trypanosomal glycolytic enzymes. Nitroblue Tetrazolium staining reveals bands at the same place as the three distinct bands in the Napthol Blue Black stain. The three distinct bands are the enzyme L-α-glycerophosphate dehydrogenase (Baranowski, 1963). The optimum pH activity for both the ammonium sulfate fraction and crude homogenate occurs at pH 6.6. A fairly broad activity range is noted from pH 5.0-pH 7.5. This broad pH optimum range is similar to the pH activity curve of the bee thorax enzyme but differs from the narrow range pH curve of the rabbit muscle enzyme (pH 7.0 - pH 7.5).

The reverse reaction (α-glycerophosphate to dihydroxyacetone phosphate) was also tested for its pH optimum of activity. A narrow range of high activity was found at pH 10.0 - 11.0. Enzyme activity decreased sharply out of this range. The narrow pH optimum of the reverse reaction has also been reported in preparations from Trypanosoma rhodesiense (Grant and Sargent, 1960). The equilibrium of this reverse reaction at hydrogen ion concentrations above 10^{-10} M favors the formation of L-α-glycerophosphate from dihydroxyacetone phosphate.

The influence of temperature on the stability of the enzyme was tested. The enzyme maintains full activity when incubated for 5 minutes at 50°C (see fig. 5). Between 60°C and 70°C all enzymatic activity is lost. The enzyme is stable at 2°C and/or at -70°C for 3-4 months.

The apparent Michaelis - Menten constant (Km) for dihydroxyacetone phosphate in the presence of saturating NADH is .3225 mM as seen in Figure 6. Dihydroxyacetone phosphate concentrations above 1.0 mM do not fit a linear Lineweaver - Burk plot. The substrate inhibition

exhibited was also found with the bee and rabbit enzyme (Baranowski, 1963; Marquardt and Brosemer, 1966 a). The enzyme fails to utilize dihydroxyacetone as a substrate.

The effects of sulfhydryl reagents on the enzyme are seen in Table 3. The enzyme is inhibited at fairly high concentrations of N-ethylmaleimide (NEM) and p-hydroxymercuribenzoate (PHMB) but is not affected by iodoacetic acid nor is activity stimulated by reduced Gluthathione (GSH). Similar effects have been noted by Grant and Sargent, for the enzyme isolated from T. rhodesiense (1956).

The Trypanocidal drug, Berenil (4,4' diamino-diazobenzene diaceturate), inhibits L-α-glycerophosphate dehydrogenase at high concentrations (50-100 μg/ml reaction volume, see Figure 7). The inhibitor concentration range is at the edge of the physiological curative dose administered (1 MCD). This in vitro data may be contrasted with a concentration of 5 μg/ml reaction volume which has recently been shown to inhibit the Escherichia coli and Micrococcus lysodeikticus DNA polymerase(s) (Pol. 1) (M. Zahalsky - personal communication). These results suggest that L-α-glycerophosphate dehydrogenase may not be a primary site of action of the drug.

The isoelectric point of the purified enzyme was obtained by isoelectric focusing in polyacrylamide gel (see Figure 8). The P_I is pH 6.4 as may also be the case for the bee L-α-glycerophosphate dehydrogenase, where crystallization by ammonium sulfate precipitation was achieved at pH 6.4. Since crystallization occurs at pH 6.4 in the bee enzyme isolation scheme, it is not surprising that the P_I should also be at this pH. The staining reaction seen at the proximal portion of the gel may be explained by the higher pH optimum of the reverse

reaction α-glycerophosphate ⟶ dihydroxyacetone phosphate. At these higher pH's a small amount of residue enzyme will stain intensely, although only trace quantities of residue enzyme are present.

DISCUSSION

The presence of L-α-glycerophosphate dehydrogenase activity in both the soluble and particulate fractions of Trypanosoma brucei cell homogenate is similarly detected in cell-free preparations of other organisms (e.g. rabbit muscle and bee thorax).

As reported for bee thorax muscle and rabbit muscle, treatment of particulate fraction(s) with a membrane solubilizing agent such as Triton X-100 releases enzyme into the supernatant. In these studies we have been unable to discern any marked differences in the properties of the enzymes within the particulate and soluble fractions. The appearance of enzyme activity in these two fractions appears to be a more general characteristic associated with other reported trypanosome enzymes, viz. aldolase, hexokinase, and acid phosphatase have been found in both the soluble and particulate fractions after differential centrifugation of cell free homogenates. Because of the uncertain localization of enzyme activity obtained in these studies it is necessary to establish a standard differential centrifugation procedure utilizing marker enzymes, as has been done for rat liver (De Duve and Berthet, 1954). Likewise, it is desirable to attempt to localize the dehydrogenase-oxidase system in Trypanosoma brucei, since cytological and biochemical evidence from other systems have shown that L-α-glycerophosphate is an intermediate in the synthesis of glycerophosphatides (Kornberg & Pricer, 1953).

The ammonium sulfate purified L-α-glycerophosphate dehydrogenase was obtained at relatively low yield, presumably because a large fraction of enzyme remained particle bound. Analysis of the purified

ammonium sulfate enzyme preparation by disc-gel electrophoresis reveals that the 50% ammonium sulfate 'cut' appears to consist mainly of the enzyme. Impurities in this enzyme preparation are seen in the disc-gel to the right of the enzyme-localized band. It is interesting to note that after storage of the 50% $(NH_4)_2\ SO_4$ enzyme preparation at -70°C the dense band which previously appeared to the right of the enzyme band dissappeared and instead one sees a faint region of contaminating proteins. However, under these same conditions L-α-glycerophosphate dehydrogenase did not exhibit any loss in activity. The high stability of this enzyme readily permits its isolation and subsequent maintenance.

The appearance of three distinct electrophoretic bands suggests that T. brucei α-glycerophosphate dehydrogenase may be present as isoenzymes. We have no further evidence on this matter and this possibility needs to be explored.

The isolated enzyme appears to have a pH optimum in the near neutral range and close to the host blood pH for the reaction: dihydroxyacetone phosphate $\longrightarrow$ L-α-glycerophosphate. At higher pH's the reverse reaction is active. From the reports of other investigators it appears that this step is not rate limiting in the overall reaction (Grant & Sargent, 1960). The observed pH optimum appears to be identical to that found for the bee thorax enzyme (pH 6.6) but different from the rabbit enzyme optimum (pH 7.5). The bimodal curve obtained with the T. brucei enzyme is also seen with the rabbit muscle enzyme (Baranowski, 1963). The reaction favoring the formation of L-α-glycerophosphate is of metabolic significance in that L-α-glycerophosphate may be used to synthesize glycerophosphatides amongst other compounds.

The K_m of .3225 mM is similar to the K_m of the enzyme isolated from the bee thorax. A Lineweaver - Burk plot reveals substrate inhibition of the rabbit, bee, and trypanosome enzyme(s). It is possible that the decrease in activity at substrate levels >1 mM may play a role in controlling the flow of glycolytic intermediates through the glycerophosphate cycle. Although the enzyme is inhibitable by PHMB and other sulfhydryl reagents, the high concentrations required to effect inhibition is similar to that found with the rabbit enzyme but contrasts with what has been reported for the bee enzyme where much lower molar concentration of PHMB inhibit enzyme activity. Since neither 10^{-2} M idoacetate nor reduced glutathione (10^{-2} M) affect the enzyme at the concentrations used, these data suggest that inhibition of trypanosome respiration by Sulfhydryl-reagents, or stimulation, as previously reported, may occur elsewhere in the glycolytic pathway (Fulton & Spooner, 1959). Because PHMB and NEM inhibit the enzyme *in vitro* (though at relatively high concentrations), it may be that the inhibitory effect *in vivo* may occur at the level of glyceraldehyde-3-phosphate. This postulated site of action remains to be tested.

Berenil inhibits L-α-glycerophosphate dehydrogenase at molar concentrations which approximate the estimated curative dose as may be given to a parasitized host. Since the observed inhibitory concentration *in vitro* appears to be at the edge of what would be a physiological level *in vivo*, it remains uncertain as to whether this enzyme site of interaction *in situ* represents a primary mode of action of the drug.

The isoelectric point, P_I, (zero *effective* charge on the protein) was calculated to be pH 6.4. This value is considered to be highly

significant in view of the fact that the bee enzyme exhibits least solubility at this point and crystallizes out of solution. This property may very likely be applied to the isolation of L-α-glycerophosphate dehydrogenase from T. brucei.

The enzyme properties reported on in this thesis (K_m, pH optimum, temperature stability, and substrate inhibition) appear in part to be similar to those obtained for the enzyme isolated from bee thoraces. Since this enzyme appears to play a key role in the metabolism of the pathogenic bloodstream trypanosomes, the studies initiated here may be extended to advance our understanding of the metabolic pathways of pathogenic trypanosomes - some of which may represent suitable targets for chemotherapeutic agents.

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